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RAPID COMMUNICATION

Pluripotent Embryonic Stem Cells from the Rat Are Capable of Producing Chimeras

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Embryonic stem cells have been enormously important in the production of targeted mutations in mice used in the study of gene function and biological aspects of disease states. The use of these cells for mouse studies is now widespread but the production of animals from similar cell lines derived from other species has not been previously reported. We demonstrate here the derivation of diploid rat embryonic stem cells (RESC-01). RESC-01 cells are SSEA-1 and alkaline phosphatase positive, grow best on primary rat embryonic fibroblasts, and can differentiate extensively *in vivo*. RESC-01 cells form cystic embryoid bodies capable of rhythmic contractions. Rat blastocysts injected with RESC-01 cells form chimeras. The results indicate that the successful *in vitro* propagation and chimera production with embryonic stem cells is not limited to the mouse. The long-term culture of rat ES cells will provide an important resource for the study of normal physiology and disease models where rat is the species of choice. © 1994 Academic Press, Inc.

The production of animals wholly derived from cultured embryonic stem (ES) cells by breeding chimeras revealed the enormous potential of ES cells for genetic analysis by combining their ability to colonize the germ line with methods for gene transfer (Bradley *et al.*, 1984), insertional mutagenesis (Zhou *et al.*, 1993), and, most recently, for targeted disruption of specific genes (Koller and Smithies, 1992). ES-like cells have been isolated from other species; however, only ES cell lines from the mouse have been brought into routine usage for genetic modification and germ line chimera production. Thus, the creation of animal models with homologous recombination using mammalian species other than the mouse is not currently possible.

Recently, our lab has developed methods for culturing preimplantation rat embryos. This has required the establishment of complex culture media and optimal culture conditions (Ng and Iannaccone, 1992; Van Winkle *et al.*, 1990). The combination of these techniques with the development of methods to reliably establish and date pseudopregnancy in the rat has allowed us to produce aggregation chimeras between two different embryos with high efficiency. By extending these techniques and combining them with procedures in use to derive and maintain mouse ES cells, we have now established the continuous culture of cells from the rat blastocyst. These rat cells, unless cultured on rat primary embryonic fibroblast feeder layers or in the presence of leukemia inhibitor factor (LIF), will differentiate extensively. Like mouse ES cells the RESC-01 (diploid rat embryonic stem cells) cells form embryoid bodies if grown in suspension and form teratomas with extensive differentiation when injected into nude mice. Most importantly, we have demonstrated that the rat cells can be used to produce chimeras by injection into rat blastocysts. Based on these criteria, we believe that these cells are the rat equivalent of mouse pluripotent embryonic stem cells. The derivation of ES cells from the rat could provide a route for the production of models for human disease, such as hypertension and heart disease, where the rat is the species of choice.

A total of 420 blastocysts from the inbred PVG strain of black hooded rats were placed on rat embryonic fibroblast feeder layers in organ culture dishes using rat embryo medium (see legend to Fig. 1). Of these, 192 blastocysts attached and after 3 days all of the attached blastocysts had expanded inner cell mass populations. These were mechanically disrupted and individually placed on new feeder layers. Mechanical disruption was repeated daily and colonies of ES-like cells were identified after 5 days. Forty-eight individual cell cultures were created in this way. We expanded the number of cells from one of these chosen at random to create the cell line RESC-01 which has a modal chromosome number of 42, XY (Fig. 1).

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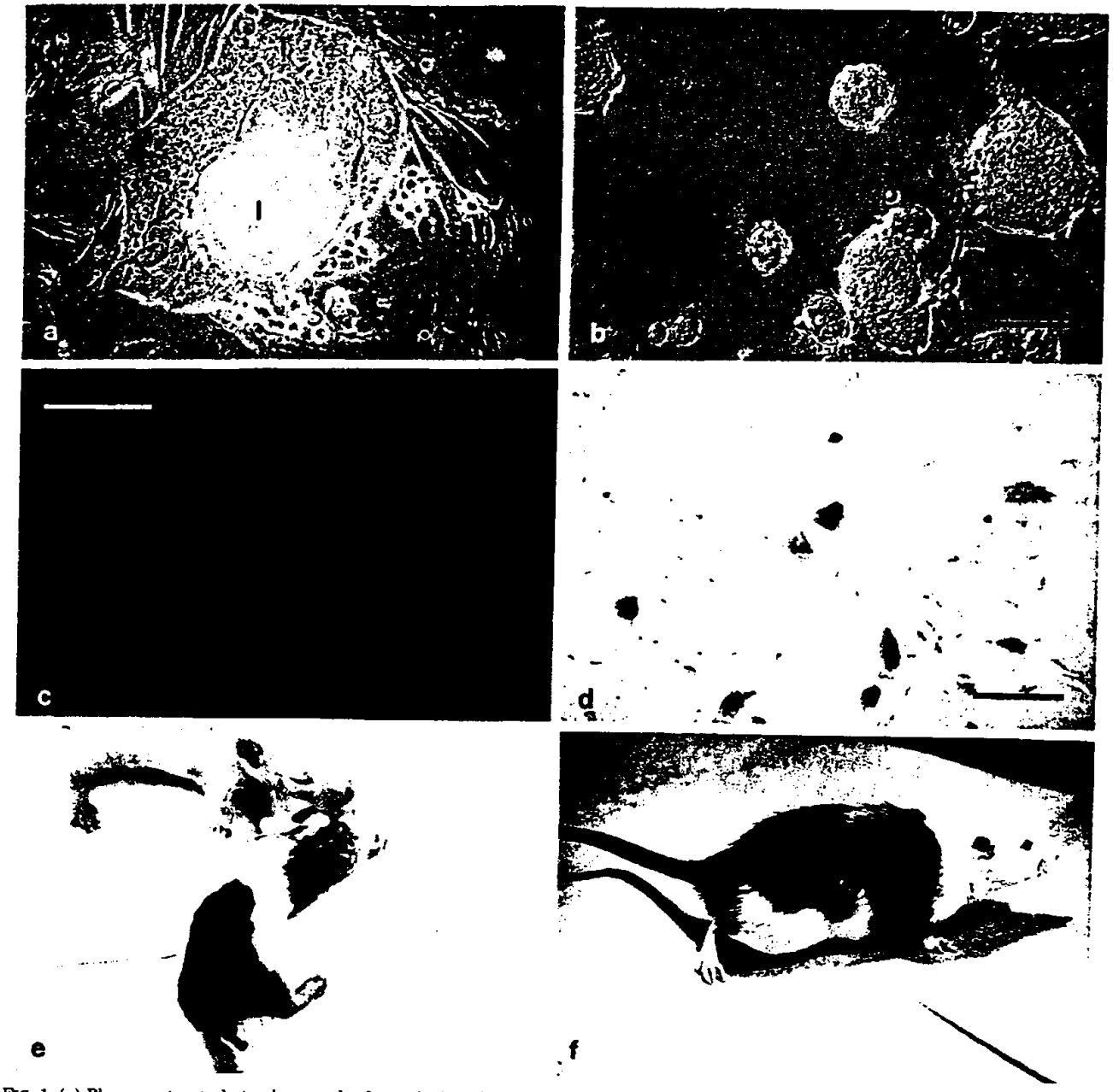

FIG. 1. (a) Phase-contrast photomicrograph of attached rat blastocysts. Rat blastocysts (PVG-*RT1*^{c} × PVG-*RT1*^{c}) were removed from the uterus by flushing with a balanced salt solution (Ng and Iannaccone, 1992). The blastocysts were placed on primary rat embryonic fibroblasts in organ culture dishes containing Markert's modification of Whittingham's medium (Yamamura and Markert, 1981) supplemented with 20% FBS (Intergen, lot screened for mouse ES cell growth), penicillin (100 units/ml)/streptomycin (100 μg/ml), 1% nonessential amino acids (NEAA, 100× stock from GIBCO, 320-1140PG), 1% nucleoside from stock (100× stock = adenosine 3.0 *M*, guanosine 3.0 *M*, cytidine 2.3 *M*, uridine 3.0 *M*, thymidine 1.0 *M*), 1% 2 β-mercaptoethanol stock (stock = 7 μl/10 ml PBS), 2 m*M* L-glutamine, and LIF (2000 units/ml Esgro, GIBCO). T, trophectoderm; I, inner cell mass; F, HREF feeder layer; bar, 100 μm. (b) Phase-contrast photomicrograph colonies of RESC-01 cells following trypsinization at passage 7 grown in DMEM supplemented as in (a); bar, 100 μm. (c) Epifluorescent photomicrograph of RESC-01 cells (passage 7) following incubation with antibodies against SSEA-1; HREF feeder layers do not react with the antibody; irrelevant first antibody controls were negative; positive controls included D3 mouse ES cells; bar, 40 μm. (d) Phase-contrast photomicrograph of RESC-01 colonies (passage 7) on HREF feeder layers and stained for alkaline phosphatase. The RESC-01 cells, but not the HREF feeder layer cells, are alkaline phosphatase positive. Similar results are obtained at passage 12; bar, 200 μm. (e) Chimera 4999 (female) photographed when 9 days old. (f) Chimera 5001 (male) photographed when 100 days old.

The growth and differentiation of RESC-01 cells were analyzed using various substrata and media. RESC-01 cells were plated at low density and grown on plastic, gelatin-coated plastic, growth-arrested STO mouse fibroblast feeder layers, and growth-arrested HREF (Holtzman strain rat embryonic fibroblast) fibroblast feeder layers. Differences in growth curves on HREF, STO, gelatin-coated plastic, and plastic were statistically significant (data not shown). Cell counts show growth was best on HREF feeder layers. In separate experiments cells were plated on several substrata or in media containing various concentrations of LIF, which has been shown to inhibit the differentiation of mouse ES cells in culture even in the absence of embryonic fibroblast feeder layers (Mummery *et al.*, 1990). When RESC-01 cells were grown in the absence of LIF or on plastic the population rapidly differentiated into cells which were morphologically distinct from undifferentiated ES cells.

The ability to maintain these cells in the undifferentiated state was compared on various substrata (Fig. 2). There was a statistically significant difference in the number of colonies which contained differentiated cells on the various substrata. On Day 2 and Day 3 following plating the least differentiation occurred on HREF, followed by STO fibroblasts and gelatin-coated plastic. Plating on plastic led to the most rapid differentiation of colonies. Growth on HREF fibroblasts or gelatin-coated plastic in the presence of various concentrations of mouse LIF demonstrated a concentration-dependent inhibition of differentiation of RESC-01 cells (Fig. 2) without affecting the rate of growth. The number of colonies in which any of the cells had differentiated was determined. The cells were plated as small clumps of 2–4 cells at low density (50,000 cells/plate) so that 50 individual colonies could be scored on the basis of morphology. Differentiation appears to be related to colony size (data not shown) and by plating single cells the undifferentiated state can be maintained for extended periods.

The spontaneous differentiation of mouse ES cells in suspension results in the formation of cystic structures which eventually develop several cell layers reminiscent of the early embryo, known as embryoid bodies. We established embryoid bodies in this manner from passage 6 RESC-01 cells. After 7 days some of the rat cystic embryoid bodies acquired complex shapes with cystic fluid filled cavities and multiple cell layers, which on histological section appeared endoderm-like and ectoderm-like. Some of these began rhythmic contractions like those produced with mouse embryoid bodies (Sanchez *et al.*, 1991). RESC-01 cells at passage 10, 11, or 12 injected into nude mice also resulted in extensive differentiation of the cells. Teratomas formed within 3 weeks which contained differentiated cells and tissues derived from all three embryonic germ layers. These included muscle, neural tissues, bone, cartilage, bone marrow, respiratory and gastrointestinal mucosa, squamous epithelium, and pancreatic acini (data not shown).

Cells isolated from cultures of undifferentiated RESC-01 cells (passage 5 or 6) were microinjected into blastocysts isolated from Day 4 pregnant Holtzman strain rats. From 10 to 30 RESC-01 cells were injected into each blastocyst and the injected blastocysts were transferred into the uteri of Holtzman albino strain female rats in their third day of pseudopregnancy (Weinberg *et al.*, 1985; Ng and Iannaccone, 1992). These procedures resulted in a pregnancy rate of 79% and a live birth rate of 39%. Eighty-nine albino pups were born and six of these displayed a patchy mixture of albino, black, and agouti coat colors (Fig. 1). These six vary from approximately 10 to 80% pigmented coat. Two are male and four are female. They all display posterior pigmentation, never seen in the PVG (hooded) or Holtzman rats. Patches of agouti and black are apparent in pigmented areas of the chimeras. Two have pigmented patches on the head, while PVG coat pattern always consists of pigmentation on the entire head. One has bilateral pigmentation on the dorsal surface of the distal forelimbs. These coat color patterns could only have occurred as a result of chimera formation between the injected RESC-01 cells and the Holtzman strain blastocyst. The patterns observed in our rat ES cell chimeras are consistent with those previously reported in aggregation chimeras formed by amalgamation of 8-cell embryos from strains genetically similar to these used here (Yamamura and Markert, 1981).

Pluripotent cells have been isolated from mink (Sukoyan *et al.*, 1992), pig (Notarianni *et al.*, 1991), and hamster (Doetschman *et al.*, 1988) but so far there are no published accounts of chimera formation with stem cells from species other than mouse. The ability to establish a stem cell population from the rat and make chimeras with them is the first step toward providing an important addition to the repertoire of genetic manipulation techniques in mammals. The creation of targeted mutations in the mouse has been a valuable source of animal models of human disease. For many diseases, however, the physiology of the mouse is either poorly understood or inappropriate for experiments germane to the particular disease. For example, the study of cardiovascular disease has extensively utilized the rat because of its small size and its appropriate physiological responses to experimental manipulations. For similar reasons the rat is important in the study of lipid transport, atherosclerosis, hypertension, and cardiomyopa-

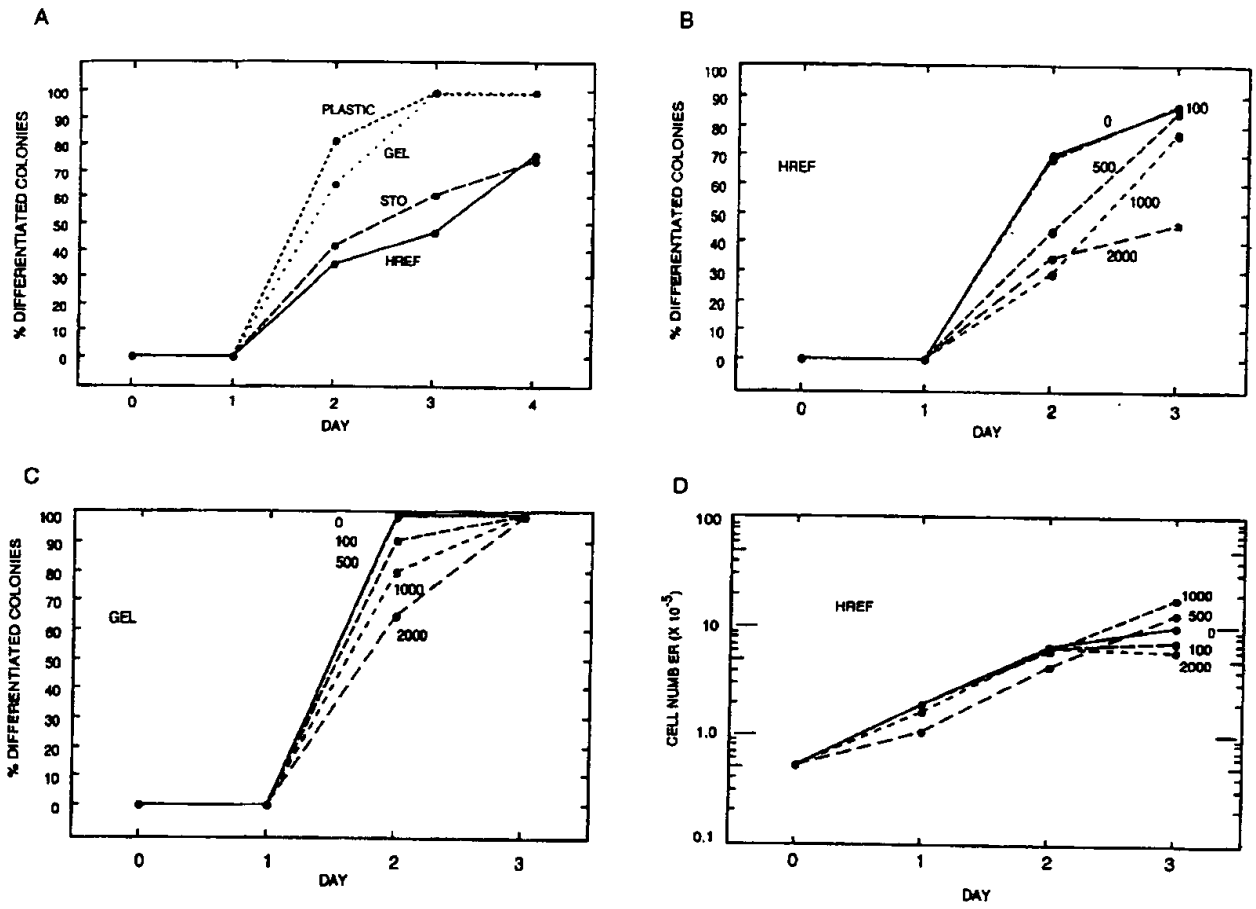

FIG. 2. (A) Differentiation of colonies of RESC-01 cells on various substrata. Standard error of the means falls within the range of the symbols. The proportion of differentiated colonies of RESC-01 cells grown on HREF is significantly different from those grown on STO on Day 2 ($P = 0.02$); from those grown on gelatin-coated plastic on Days 2, 3, and 4 ($P < 0.001$); and from those grown on plastic on Days 2, 3, and 4 ($P < 0.001$). The proportion of differentiated colonies of RESC-01 cells grown on STO is significantly different from those grown on gelatin-coated plastic on Days 2, 3, and 4 ($P < 0.001$) and from those grown on plastic on Days 2, 3, and 4 ($P < 0.001$). The proportion of differentiated colonies of RESC-01 cells grown on gelatin-coated plastic is significantly different from those grown on plastic on Day 2 ($P < 0.003$). (B and C) Differentiation of RESC-01 colonies on HREF fibroblasts or gelatin-coated plastic. Fifty colonies were counted in six determinations. The numbers by each curve represent the concentration of mouse LIF in units/ml. The proportion of differentiated colonies of RESC-01 cells grown of HREF in the absence of LIF is significantly different in the presence of 500 units/ml LIF on Days 2 and 3 ($P < 0.001$); in the presence of 1000 units/ml LIF on Day 2 ($P < 0.001$); and in the presence of 2000 units/ml LIF on Days 2 and 3 ($P < 0.006$). The proportion of differentiated colonies of RESC-01 cells grown on gelatin-coated plastic in the absence of LIF from those grown in the presence of 500 units/ml of LIF on Day 2 ($P < 0.0001$); in the presence of 1000 units/ml of LIF on Day 2 ($P = 0.007$); and in the presence of 2000 units/ml of LIF on Day 2 ($P = 0.0001$). Cells were prepared by trypsinization and plated on the indicated substrata at passage 6. The curves were produced by plating 50,000 cells as clumps of 2-3 cells each on 10^6 growth-arrested embryonic fibroblasts. Fifty colonies were scored in six determinations made on duplicate plates. Any differentiated cells (epithelial, mesenchymal, or endodermal morphologies) in a colony caused the colony to be scored as differentiated. (D) Growth curves in the presence of various concentrations of LIF. LIF did not influence proliferation of these cells.

thy. Detailed information from behavioral studies are much more readily available in the rat than in the mouse. Therefore, the availability of ES cells from the rat, by extending the scope of studies to include targeted mutational analysis, will be critical to furthering our understanding of a variety of disease states. Nevertheless, the probability of obtaining an undifferentiated continuously proliferating cell line is certainly less than in the mouse. Strain differences in the frequency of establishing continuous ES cell lines in the mouse are well known, and although it is reasonable to suppose that this is also true in the rat, at this time we have no formal evidence that it is. These are important considerations since as yet our chimeras have not demonstrated germ line transmission. While several of the animals reported here have shown limited reproductive capacity, it is

likely that additional cell lines will have to be established in order to fully utilize ES cell technologies in the rat. By extending the studies reported here and by continuing to work with species other than the mouse it will be possible to resolve many of these issues and bring the valuable genetic resources of ES cell technology to other mammalian species.

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